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Anthrax Lethal Toxin Impairs CD1d-Mediated Antigen Presentation by Targeting the Extracellular Signal-Related Kinase 1/2 Mitogen-Activated Protein Kinase Pathway▽

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Lethal toxin (LT) is a critical virulence factor of *Bacillus anthracis* and an important means by which this bacterium evades the host's immune system. In this study, we demonstrate that CD1d-expressing cells treated with LT have reduced CD1d-mediated antigen presentation. We earlier showed an important role for the mitogen-activated protein kinase extracellular signal-regulated kinase 1/2 (ERK1/2) in the regulation of CD1d-mediated antigen presentation, and we report here that LT impairs antigen presentation by CD1d in an ERK1/2-dependent manner. Similarly, LT and the ERK1/2 pathway-specific inhibitor U0126 caused a decrease in major histocompatibility complex (MHC) class II-mediated antigen presentation. Confocal microscopy analyses revealed altered intracellular distribution of CD1d and LAMP-1 in LT-treated cells, similar to the case for ERK1/2-inhibited cells. These results suggest that *Bacillus anthracis* has the ability to evade the host's innate immune system by reducing CD1d-mediated antigen presentation through targeting the ERK1/2 pathway.

The Gram-positive bacterium *Bacillus anthracis* is the causative agent of anthrax and can kill its host after only a few days (12, 14, 15, 23). Lethal toxin (LT), composed of lethal factor (LF) and protective antigen (PA), is a major virulence factor produced by this bacterium (5, 24). PA is involved in cell binding, whereas LF possesses the enzymatic toxic activity (5, 8, 24). PA binds to cell surface receptors called anthrax toxin receptors (ATRs) and facilitates the entry of LF into the host cell (1, 7). LF is a metalloprotease responsible for impairing mitogen-activated protein kinase (MAPK) signaling (8).

The MAPK signaling pathways are mediated by three different serine/threonine protein kinases: extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK) (11, 28). Each of these signaling pathways plays an important role in the innate and adaptive immune responses (13, 37). A number of bacterial pathogens manipulate signaling pathways to affect the immune system of the host (6). *Bacillus anthracis* via LT is capable of disrupting MAPK signaling pathways that are important for dendritic cell and macrophage function (2, 3, 32). These pathways include those we have shown to regulate CD1d-mediated antigen presentation (10, 20, 25, 34).

CD1d molecules are structurally similar to major histocompatibility complex (MHC) class I molecules, but their intracellular trafficking is similar to that of MHC class II molecules (9, 36). MHC class I and class II molecules present peptide antigens to T cells, whereas CD1d molecules present lipid antigens to a unique subset of T cells called natural killer T (NKT) cells (9). Various pathogens inhibit CD1d-mediated antigen presentation in order to evade the host's innate immune system by altering cell signaling pathways (25, 34). Because NKT cells are an important component of the innate immune system and act as a bridge between innate and adaptive immune responses, we analyzed the effect of LT on CD1d-mediated antigen presentation. Our results suggest that LT impairs CD1d-mediated antigen presentation in an ERK1/2-dependent manner.

MATERIALS AND METHODS

Mice. Female BALB/c and C3H/HeN mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and used at 6 to 8 weeks of age. All procedures were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

Cell lines and other reagents. Murine LMTK-CD1d1 and L-CD1-DR4 cells and the mouse CD1d-specific NKT cell hybridomas DN32.D3 (type I), N37-1A12 (type II), and N38-2C12 (type I) were cultured as described previously (19). TA3 B lymphoma cells and 3A9 and 17.9 T cell hybridomas were used as previously described (19). Purified and biotinylated monoclonal antibodies (MAb) specific for murine interleukin-2 (IL-2) and gamma interferon (IFN-γ) were purchased from BD Biosciences (San Diego, CA). Recombinant mouse IL-2, IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-4 were obtained from PeproTech (Rocky Hill, NJ). Both the ERK1/2 inhibitor (U0126) and ERK1/2-specific antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Recombinant anthrax protective antigen (PA) and lethal factor (LF) were purchased from List Biological Laboratories, Inc. (Campbell, CA). Chloroquine was purchased from Sigma-Aldrich (St. Louis, MO).

Generation of BMDCs. Bone marrow cells from BALB/c mice were cultured in the presence of 10 ng/ml each of GM-CSF and IL-4 as previously described (10). On day 7, the plates were gently flushed (three or four times) to remove the loosely adherent cells, which were subsequently used in analyses as bone marrow-derived dendritic cells (BMDCs). For MHC class II-mediated antigen presentation, BMDCs were generated from C3H/HeN mice and were grown for 6 days in the presence of GM-CSF and IL-4. On day 6, BMDCs were treated with lipopolysaccharide (LPS) overnight and used to analyze MHC class II-mediated antigen as described previously (19).

NKT cell coculture assays. LMTK-CD1d1 cells or BMDCs were treated with various concentrations of LF (0.1, 0.5, and 1.0 μg/ml) with or without a fixed concentration of PA (1.0 μg/ml) for 24 h. The cells were then washed, fixed, and cocultured with the indicated NKT cell hybridomas, and IL-2 production was measured as described previously (26). For fresh NKT cells, liver mononuclear cells from BALB/c mice were harvested as previously described (10) and cocultured with LT-treated BMDCs for 48 h. IFN-γ production by NKT cells was measured by enzyme-linked immunosorbent assay (ELISA) (10). To analyze the effect of chloroquine on LT-induced reduction of CD1d-mediated antigen presentation, LMTK-CD1d1 cells were treated with LT (PA [1 μg/ml] plus LF [1

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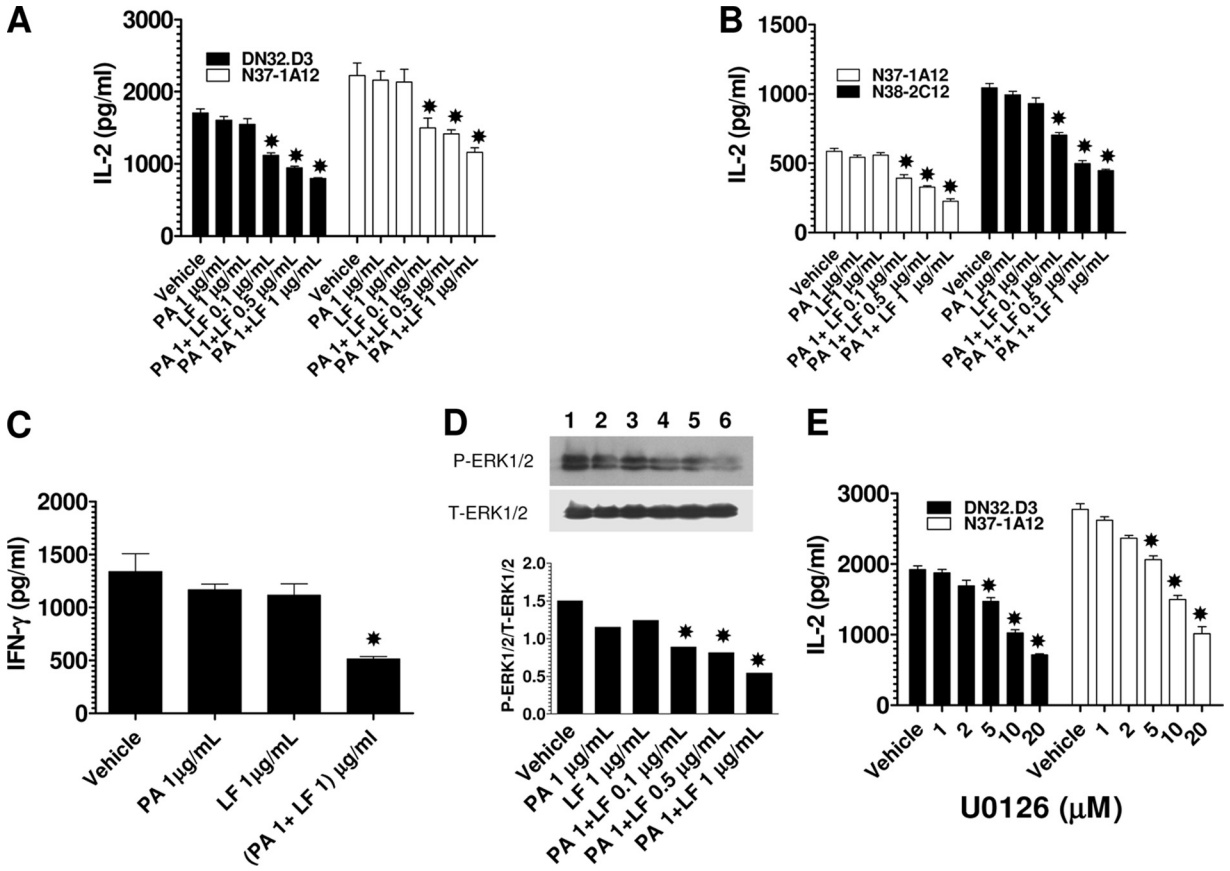

FIG. 1. Anthrax lethal toxin inhibits CD1d-mediated antigen presentation in an ERK/12-dependent manner. (A and B) LMTK-CD1d1 cells (A) or BMDCs (B) were treated with vehicle, PA, LF, or LT as indicated, washed, fixed, and cocultured with the indicated NKT cell hybridomas. IL-2 production was measured by ELISA. *, $P < 0.001$ compared to vehicle. (C) BMDCs were treated with vehicle or LT for 24 h, washed, fixed, and cocultured with primary NKT cells for 48 h. Supernatants were harvested to measure IFN-γ. *, $P < 0.001$ compared to vehicle treatment. (D) LMTK-CD1d1 cells were treated with vehicle or LT as described above, and the levels of phospho- and total ERK1/2 were analyzed by Western blotting. Lanes: 1, vehicle; 2, PA (1 μg/ml); 3, LF (1 μg/ml); 4, PA (1 μg/ml) plus LF (0.1 μg/ml); 5, PA (1 μg/ml) plus LF (0.5 μg/ml); 6, PA (1 μg/ml) plus LF (0.5 μg/ml). *, $P < 0.001$ compared to vehicle. (E) LMTK-CD1d1 cells were treated with vehicle or the ERK1/2 pathway-specific inhibitor U0126 (1, 2, 5, 10, or 20 μM) for 24 h, washed, fixed, and cocultured with the indicated NKT cell hybridomas. *, $P < 0.001$ compared to vehicle. Error bars indicate standard deviations.

μg/ml]) in the presence (10 μM) or absence of chloroquine for 24 h, fixed, and cocultured with NKT cells as described above.

MHC class II antigen presentation assay. Murine B cell lymphoma TA3 cells or C3H/HeN BMDCs were treated with PA (1 μg/ml), LF (1 μg/ml), LT (PA plus LF), or various concentrations (0, 2.5, 5.0, 10, and 20 μM) of U0126 in the presence of 2 mg/ml hen egg lysozyme (HEL). The cells were then washed, fixed, and cocultured with the 3A9 T cell hybridoma to measure IL-2 production as described previously (19). Similarly, L-CD1d1-DR4 cells were treated with LT or the ERK1/2 pathway inhibitor U0126 in the presence of 10 μM human serum albumin (HSA). The cells were then washed, fixed, and cocultured with the 17.9 T cell hybridoma as described previously (19).

Western blotting. LMTK-CD1d1 cells were treated with vehicle or the indicated concentrations of PA, LF, or LT (PA plus LF). The cells were lysed in lysis buffer containing Complete protease inhibitor tablets (Roche Diagnostics, Indianapolis, IN) as described previously (20). Equal amounts of protein were loaded into each well, resolved on a 10% SDS-polyacrylamide gel, and subsequently transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Antibodies specific for the indicated proteins were used to detect the respective bands, which were developed by chemiluminescence before exposure on film (25).

Confocal microscopy. LMTK-CD1d1 cells plated in collagen-coated sterile glass-bottom 35-mm dishes (MatTek, Ashland, MA) were treated with the indicated concentrations of LT or U0126 for 24 h at 37°C. The cells were stained for CD1d and LAMP-1, followed by fluorescein isothiocyanate (FITC)- and Texas red-conjugated secondary antibodies, for analysis by confocal microscopy as previously described (26, 29). The percent colocalization of CD1d and LAMP-1 was determined using MetaMorph software (version 5; Molecular Devices, Sunnyvale, CA), in which six random fields were chosen from each picture.

Statistical analysis. The data were analyzed by a one-way analysis of variance (ANOVA) with a Bonferroni posttest using GraphPad PRISM software (version 5.0 for Windows; GraphPad, San Diego, CA). A P value of below 0.05 was considered significant. All experiments were performed three to five times.

RESULTS AND DISCUSSION

Anthrax lethal toxin impairs CD1d-mediated antigen presentation by inhibiting ERK1/2. In order to analyze the effect of LT on CD1d-mediated antigen presentation, murine LMTK-CD1d1 cells were treated with various concentrations of LF with or without a fixed concentration of PA (1 μg/ml) and cocultured with NKT cells. LMTK-CD1d1 cells treated with LT (PA plus LF) showed a concentration-dependent reduction in CD1d-mediated antigen presentation to either type I (e.g., DN32.D3) or type II (e.g., N37-1A12) NKT cells, as determined by decreased IL-2 production by the CD1d-specific NKT cells (Fig. 1A). To determine the effect of LT on CD1d-mediated antigen presentation by primary antigen-presenting

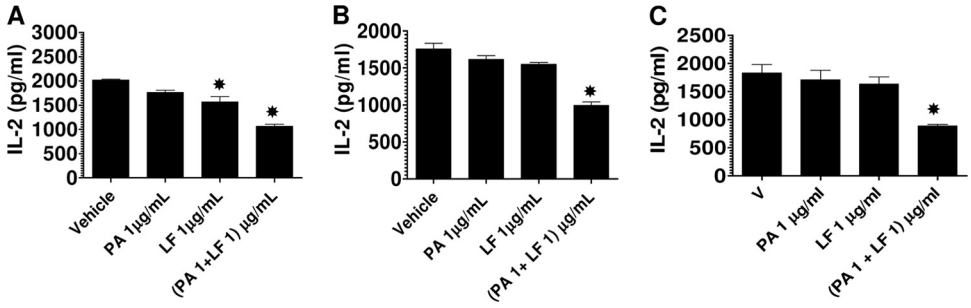

FIG. 2. Anthrax lethal toxin impairs MHC class II-mediated antigen presentation. (A) TA3 cells were treated with vehicle or LT in the presence of HEL, fixed, and cocultured with the 3A9 T cell hybridoma. *, $P < 0.001$ compared to vehicle. (B) L-CD1d1-DR4 cells were treated with vehicle or LT in the presence of HSA, fixed, and then cocultured with the 17.9 T cell hybridoma. *, $P < 0.001$ compared to vehicle. (C) BMDCs from C3H/HeN mice were treated with vehicle, PA, LF, or LT in the presence of HEL; fixed; and cocultured as described above. The amount of IL-2 in the supernatant was measured by ELISA. *, $P < 0.001$ compared to vehicle. Error bars indicate standard deviations.

cells, BMDCs were treated with LT as described above. As found with LMTK-CD1d1 cells, LT also caused a reduction in CD1d-mediated antigen presentation by BMDCs (Fig. 1B). LT did not alter the cell surface expression of CD1d on either LMTK-CD1d1 cells or BMDCs (data not shown). Prior studies have shown that LT can be toxic to GM-CSF-induced BMDCs under various conditions (2, 4). In all experiments performed for this study, our BMDCs were generated using GM-CSF plus IL-4, thus likely accounting for the high cell viability observed at 24 h after LT-treatment (data not shown). BMDCs treated with LT were also cocultured with primary NKT cells. LT caused a reduction in CD1d-mediated antigen presentation to primary NKT cells as well (Fig. 1C). These results suggest that *B. anthracis* evades the innate immune system by impairing CD1d-mediated antigen presentation.

We have shown that the ERK1/2 MAPK pathway plays an important role in the regulation of CD1d-mediated antigen presentation (25). Further, it has been reported that LT impairs MAPK signaling (32). Thus, it was possible that the reduction in antigen presentation following LT treatment was due to changes in ERK1/2 activation. To test this, LMTK-CD1d1 cells were exposed to vehicle or LT, and the status of ERK1/2 phosphorylation was analyzed. LT substantially reduced ERK1/2 phosphorylation (Fig. 1D). Consistent with our prior report (25), LMTK-CD1d1 cells treated with various concentrations of the ERK1/2 pathway-specific inhibitor U0126 were impaired in CD1d-mediated antigen presentation (Fig. 1E). Interestingly, MAPKs play an important role in T-cell receptor (TCR)-mediated signaling for optimum T cell activation (37), and anthrax toxin causes functional anergy in NKT cells by disrupting TCR signaling (17). MAPKs also regulate CD1d-mediated antigen presentation by altering the intracellular trafficking and distribution of CD1d (25). As previously reported for other systems (2, 32), it was found that treatment of LMTK-CD1d1 cells with LT caused a reduction in ERK1/2 phosphorylation (Fig. 1D). The inhibitory effect of LT on both CD1d-mediated antigen presentation and ERK1/2 phosphorylation is consistent with our earlier finding that blocking the ERK1/2 pathway reduces CD1d-mediated antigen presentation (25). This suggests that LT has the ability to affect the innate immune system by reducing CD1d-mediated antigen presentation through targeting ERK1/2.

LT impairs MHC class II-mediated antigen presentation. It has been previously reported that LT-treated dendritic cells were impaired in the priming of MHC class II-restricted antigen-specific $CD4^+$ T cells and in the expression of costimulatory molecules such as CD40, CD80, and CD86 (2). MHC class II molecules traverse through some of the same endocytic compartments as CD1d (18, 26). Because LT reduced CD1d-mediated antigen presentation, it was likely that antigen presentation by MHC class II would be altered as well. Therefore, to analyze the effect of LT on MHC class II-mediated antigen presentation, mouse TA3 B cell lymphoma cells were treated with LT in the presence of hen egg lysozyme (HEL) and cocultured with the 3A9 T cell hybridoma (19). As observed with CD1d-mediated antigen presentation, LT treatment also reduced antigen presentation by MHC class II molecules without altering its cell surface expression (Fig. 2A and data not shown). However, unlike what was observed with CD1d, LF alone was able to significantly decrease MHC class II-mediated antigen presentation. In another experiment, HLA-DR4-expressing L-CD1d1 (L-CD1-DR4) cells were treated with LT to test presentation of human serum albumin (HSA) to the 17.9 T cell hybridoma. LT also impaired MHC class II-mediated antigen presentation in this system (Fig. 2B). To analyze the effect of LT on MHC class II-mediated antigen presentation by primary cells, BMDCs from C3H/HeN mice were treated with or without LT in the presence of HEL and cocultured with the 3A9 T cell hybridoma as previously described (19). Similar to its effect on TA3 cells, LT also reduced MHC class II-mediated antigen presentation by BMDCs (Fig. 2C). This suggests that the functional expression of both CD1d and MHC class II molecules is altered by LT and confirms a previous study analyzing the latter antigen presentation system (2). These data are also consistent with our prior reports suggesting that MHC class II- and CD1d-mediated antigen presentations are similarly regulated by specific cell signaling pathways (10, 19, 20).

Inhibition of the ERK1/2 pathway reduces MHC class II-mediated antigen presentation. ERK1/2 has also been shown to regulate intracellular trafficking, and its inhibition causes intracellular accumulation of endosomal cargo (27). Endosomal localization of the MEK1-ERK pathway is required for full activation of ERK1/2, and disruption of the endosomal ERK signaling results in aberrant subcellular distribution and trafficking of late endosomes (31, 33). We have earlier shown that inhibition of ERK1/2 reduces CD1d-mediated antigen presentation by altering the intracellular distribution of CD1d (25). ERK1/2 has been shown to regulate the class II activator

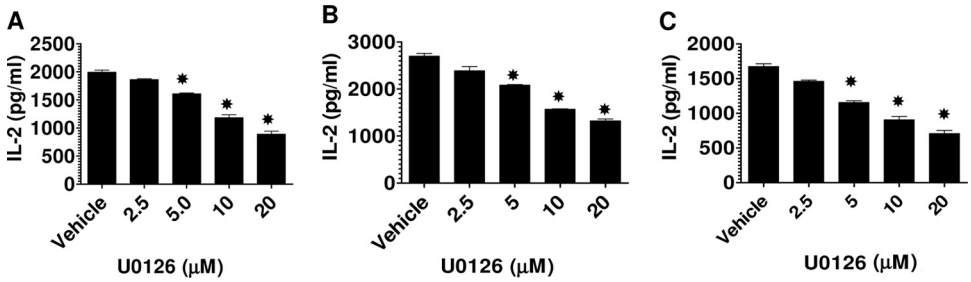

FIG. 3. Inhibition of ERK1/2 impairs MHC class II-mediated antigen presentation. (A) TA3 cells were treated with vehicle or the indicated concentrations of U0126 in the presence of HEL, fixed, and cocultured with the 3A9 T cell hybridoma. *, $P < 0.001$ compared to vehicle. (B) L-CD1d1-DR4 cells were treated with vehicle or the indicated concentrations of U0126 in the presence of HSA, fixed, and then cocultured with the 17.9 T cell hybridoma. *, $P < 0.001$ compared to vehicle. (C) BMDCs from C3H mice were treated with vehicle or the indicated concentrations of U0126 in the presence of HEL, fixed, and cocultured as described above. The amount of IL-2 in the supernatant was measured by ELISA. *, $P < 0.001$ compared to vehicle. Error bars indicate standard deviations.

CIITA (33). To analyze the effect of ERK1/2 inhibition on MHC class II-mediated antigen presentation, TA3 cells were treated with various concentrations of U0126 in the presence of HEL and cocultured with the 3A9 T cell hybridoma as described earlier (19). Inhibition of the ERK1/2 pathway by U0126 caused a concentration-dependent reduction in antigen presentation by MHC class II molecules (Fig. 3A). In another experiment, L-CD1-DR4 cells were treated with U0126 in the presence of HSA and cocultured with the 17.9 T cell hybridoma as described previously (19). U0126 also impaired MHC class II-mediated antigen presentation by L-CD1-DR4 cells (Fig. 3B). To analyze the effect of U0126 on primary antigen-presenting cells, C3H/HeN BMDCs were treated with various concentrations of U0126 in the presence or absence of HEL and cocultured with the 3A9 T cell hybridoma. U0126 also caused inhibition of MHC class II-mediated antigen presentation by BMDCs in a concentration-dependent manner (Fig. 3C). This suggests that the ERK1/2 signaling pathway regulates the function of MHC class II molecules.

Anthrax lethal toxin alters the intracellular localization of CD1d. CD1d molecules are likely loaded in late endocytic compartments (26), and we have shown that inhibiting the ERK1/2 pathway changes the intracellular distribution of CD1d in these compartments (25). Thus, as LT inhibited the phosphorylation of ERK1/2 and caused a reduction in CD1d-mediated antigen presentation without a change in CD1d cell surface levels, this effect might have been due to intracellular changes in CD1d distribution. To determine if LT could indeed change the intracellular location of CD1d, LMTK-CD1d1 cells were treated with or without LT and then the colocalization of CD1d with the late endosome/lysosome marker LAMP-1 was analyzed by confocal microscopy. U0126 served as a control, as we have already shown that inhibiting the ERK1/2 pathway decreases the colocalization of CD1d and LAMP-1 (25). A significant decrease in the colocalization of CD1d and LAMP-1 was observed in cells treated with LT (i.e., PA plus LF) (Fig. 4A and B). These data are consistent with

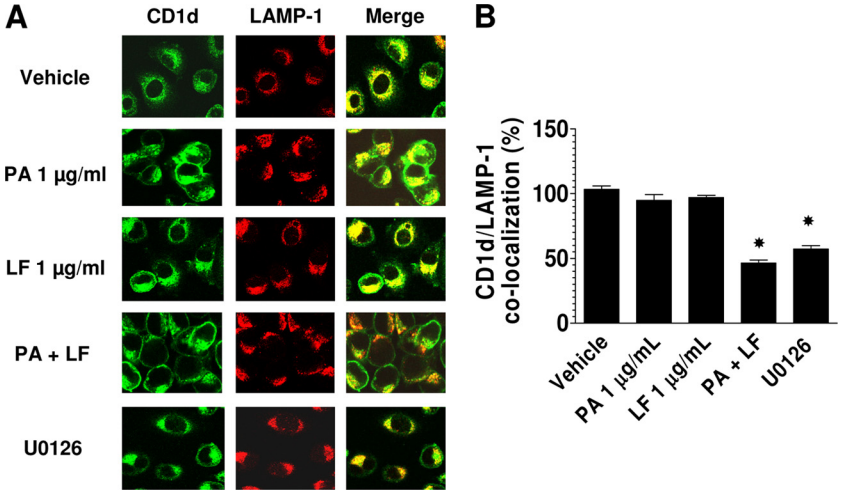

FIG. 4. Treatment with LT alters the intracellular localization of CD1d. LMTK-CD1d1 cells were treated with vehicle, PA (1 μg/ml), LF (1 μg/ml), LT (PA plus LF at 1 μg/ml each), or U0126 (10 μM) as described in the legend to Fig. 1. The cells were then washed, fixed, stained for CD1d (green) and LAMP-1 (red), and analyzed by confocal microscopy (A), and six random fields were chosen to calculate the percent colocalization (B). *, $P < 0.001$ compared to vehicle alone. Error bars indicate standard deviations.

the hypothesis that LT and U0126 impair antigen presentation by CD1d by altering its intracellular distribution.

Various pathogens use the endocytic pathway for entry into the host cells, and anthrax toxin requires the acidic pH in endocytic compartments for its enzymatic activity (16). Thus, not surprisingly, we found that chloroquine reduces the activity of lethal toxin by preventing endosomal acidification and thereby reverses its effect on CD1d-mediated antigen presentation (data not shown). During an anthrax infection, would it be possible that bacterial lipids are being presented by CD1d? We and others have shown that lipids derived from *Sphingomonas* can stimulate NKT cells in a CD1d-dependent manner (21, 22, 30, 35). Perhaps in the anthrax case, were this to be true, the lipids might instead be inhibitory. Because endocytic compartments are required for the processing and loading of ligands onto CD1d (9), the inhibition of ERK1/2 may play an important role in altering the distribution of CD1d molecules in these intracellular compartments. An anthrax infection could thus reduce lipid antigen loading of CD1d in those compartments by affecting intracellular trafficking as a result of ERK1/2 inhibition by LT. This is supported by our prior report showing impaired CD1d-mediated antigen presentation in cells with reduced ERK1/2 activation (25). Therefore, these results suggest that ERK1/2 plays a critical role in the endosomal trafficking of CD1d molecules, allowing these molecules to be loaded with the proper ligands to activate NKT cells. Overall, the results reported here strongly suggest that the anthrax lethal toxin targets this signaling pathway as a means to impair antigen presentation by CD1d in the mouse model.

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